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Salinity Variation in a Mangrove Ecosystem: A Physiological Investigation to Assess Potential Consequences of Salinity Disturbances on Mangrove Crabs

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Dimitri Theuerkauff, Georgina A. Rivera-Ingraham, Jonathan A.C. Roques, Laurence Azzopardi, Marine Bertini, Mathilde Lejeune, Emilie Farcy, Jehan-Hervé Lignot, and Elliott Sucré (2018) Salinity is one of the main environmental factors determining coastal species distribution. However, in the specific case of mangrove crabs, salinity selection cannot be understood through ecological approaches alone. Yet understanding this issue is crucial in the context of mangrove conservation, since this ecosystem is often used as biofilter of (low-salinity) wastewater. Crabs are keystone species in this mangrove ecosystem and are differentially affected by salinity. We hypothesize that crab salinity selection may be partly explained by specific salinity-induced physiological constraints associated with osmoregulation, energy and redox homeostasis. To test this, the response to salinity variation was analysed in two landward mangrove crabs: the fiddler crab *Tubuca urvillei*, which inhabits low-salinity areas of the mangrove, and the red mangrove crab *Neosarmatium meinerti*, which lives in areas with higher salinity. Results confirm that both species are strong hypo-/hyper-osmoregulators that deal easily with large salinity variations. Such shifts in salinity do not induce changes in energy expenditure (measured as oxygen consumption) or in the production of reactive oxygen species. However, *T. urvillei* is physiologically suited to habitats with brackish water, since it presents i) high hemolymph osmolalities over a wider range of salinities and lower osmoregulatory capacity in seawater, ii) high Na^+/K^+-ATPase (NKA) activity in the posterior osmoregulatory gills and iii) a thicker osmoregulatory epithelium along the posterior gill lamellae. Therefore, while environmental salinity alone cannot directly explain fiddler and red mangrove crab distributions, our data suggest that salinity selection is indeed influenced by specific physiological adjustments.

Key words: Bioenergetics, Osmoregulation, Salinity-induced oxidative stress, Mangrove, Decapods.

BACKGROUND

Mangroves are key ecosystems (Waycott et al. 2011; Ellison 2015) that are decreasing at an alarming rate despite their ecological value (Giri et al. 2011). In tropical economies across

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the world, mangrove forests are often used as biofilters for domestic wastewater (Wong et al. 1997; Ouyang and Guo 2016 2018; Capdeville et al. 2018; Theuerkauff et al. 2018). This does not negatively impact the mangroves themselves, but their associated macrofauna is frequently affected. Among the latter, burrowing crabs are keystone mangrove species that play a critical role in the maintenance of mangrove forest health through their contribution to bioturbation and organic matter degradation (Smith III et al. 1991; Lee 1998). These processes are essential in a mangrove forest, ensuring matter cycling and the maintenance of sediment biochemical heterogeneity (Emmerson and McGwynne 1992; Kristensen 2008; Penha-Lopes et al. 2009). To understand crab distribution within mangroves, many studies have addressed the impact of environmental factors such as food availability, sediment characteristics, pore water availability, canopy density, site height, sediment mounds, soil penetrability and soil surface temperature (Bezerra et al. 2006; Salgado-Kent and McGuinness 2010; Nobbs and Blamires 2015 2016). However, salinity variation is usually a key parameter determining distribution in tropical estuaries, even for euryhaline species (Blaber 1997). Moreover, salinity changes and amplitudes are increased by domestic wastewater discharges and climate change (Alongi 2008; Gilman et al. 2008). In this sense, understanding variabilities in tolerance to salinity variation and the associated ecophysiological factors is a key factor in the protection of mangrove-associated macrofauna. This approach will contribute to mangrove forest conservation in the long term.

Much work has already been done on the effect of salinity variation on crab physiology, namely osmoregulation (Gilles et al. 1988; Anger and Charmantier 2000; Cieluch et al. 2004; Garçon et al. 2009; Lignot and Charmantier 2015). However, some authors suggest that mangrove crab distribution is not strongly linked to their physiological ability to tolerate salinity and that neither salinity tolerance nor osmoregulatory ability adequately explain the zonation patterns observed in the field (Frusher et al. 1994; Gillikin et al. 2004). The aim of this study is therefore to provide a more detailed description of this physiological approach with a bioenergetic perspective. This has already been suggested as a valid marker of invertebrate vulnerability to environmental stress (Sokolova et al. 2012) and identified as a means to ultimately evaluate if these physiological capacities are closely linked with crab habitats.

Exposure to salinity variation requires organisms to implement the necessary and energy-consuming mechanisms to counteract ion and water fluxes. Among other things, this involves significant functional, morphological and ultrastructural modifications of specialized osmoregulatory tissues and cells (Compere et al. 1989; Pequeux 1995). Mitochondria are the powerhouses of the cell and are responsible for fueling these processes. However, their activity also results in the production of reactive oxygen species (ROS) which, if uncontrolled, cause what is commonly known as “oxidative stress” - *i.e.* damage to proteins, lipids and/ or nucleic acids (Sies 1985) - and eventually lead to mutagenesis or even cell death. Exposure to salinity variations, as with many other biotic and abiotic stressors, may cause increased production of ROS. Therefore, salinity acclimation and its effects on the hydromineral balance, oxygen consumption rate and redox balance (at both the gill and animal levels) have attracted some attention in recent years, notably in crabs (Freire et al. 2011; Paital and Chainy 2012; Rivera-Ingraham et al. 2016a) and other intertidal invertebrates (Rivera-Ingraham et al. 2016b).

From a physiological perspective, we investigated the tolerance of two mangrove crab species to salinity variation in Mayotte, one of the main islands of the Comoros archipelago (Mozambique Channel) where 1.8% (703 ha) of the land surface is covered by mangrove forests (Jeanson et al. 2014). The study focuses on two dominant species with bimodal breathing capacities, both distributed in the upper part of the mangrove but in slightly different areas. This determines their exposure to significantly different environmental conditions, notably in terms of salinity variation. We considered the red mangrove crab *Neosarmatium meinerti* (Sesarmidae, de Man, 1887), which is most frequently found in areas exposed to drastic salinity variations ranging from hypersaline conditions (during low tide and the dry season) to freshwater (derived from upstream run-offs during the rainy season). The second species studied is the fiddler crab *Tubuca urvillei* (Ocypodidae, H Milne Edwards, 1852), which more frequently colonizes open areas surrounding freshwater streams. Unlike *N. meinerti*, *T. urvillei* uses a burrow-plugging behavior at high tide (a common behavior in other fiddler crabs) to avoid full immersion and hypoxemia (Fusi et al. 2015). By studying these two species with different habitat use within the mangrove forest, we demonstrate

that the physiological response to salinity variation (in terms of osmoregulation, associated bioenergetics and redox homeostasis) partly explains their distribution.

MATERIALS AND METHODS

Study site, animal collection and maintenance

Neosarmatium meinerti and *Tubuca urvillei* were collected during the rainy season from the Malamani mangrove (Chirongui bay, Mayotte, 12°55'1"S, 45°9'23") in the Comoros archipelago (Fig. 1A). *Neosarmatium meinerti* were collected in the upper part of the mangrove that is composed of spurred (*Ceriops tagal*) and white (or grey) mangrove trees (*Avicennia marina*), beneath which *N. meinerti* burrows are abundant. *Tubuca urvillei* were collected along the banks of a freshwater (FW) rivulet where trees are absent (Fig. 1B-E). These two areas can be totally submerged by seawater (SW) at high tide (Fig. 1D) and can also be flooded by FW at low tide after heavy rainfall (Fig. 1F). The collection of these crabs was restricted to the banks close to spurred and red mangrove trees (*Rhizophora mucronata*). All animals were collected at low tide, and water samples were taken from random burrows for verification of osmolality for each species. Crabs of similar fresh body mass were transferred to the experimental facility of the University Center of Mayotte (CUFR). They were placed in individual plastic boxes with approximate dimensions of 16 × 11 × 7 cm (l × w × h). Two-thirds of each box was filled with SW (salinity ~33 PSU; 970 mOsmol·kg^{-1}). Crabs were then acclimated for 3 days at 28 ± 1°C, the average water temperature recorded in Mayotte (Ballorain et al. 2013). Boxes were connected to a closed recirculation water system. Each system consisted of 24 (4 × 6) boxes connected to an 80 l water reservoir. A water pump containing a biofilter ensured the supply of clean and aerated SW to each box.

Experimental conditions

For all experiments, males and females of *N. meinerti* were used in the same proportion (fresh body weight, FBW: 35 ± 8 g) (Fig. 2A). However, for *T. urvillei*, only males (FBW: 6.9 ± 1.7 g) were considered (Fig. 2B) due to the large weight difference between sexes. All animals were in intermolt stage, verified *a-posteriori* through epipodite examination.

After the initial 3-day acclimation period in SW (Fig. 2C), a first batch of crabs was exposed to different salinities to determine the osmolality curve and the isosmotic point (*i.e.* the environmental salinity at which the osmolality of body fluids and environmental media are equal) for each species. To conduct the energy-redox analyses (respirometry and ROS measurements), a second batch of animals was distributed among 3 salinities: SW, diluted seawater (dSW, 294 mOsm·kg^{-1}, salinity ~10 PSU), and isosmotic seawater (iSW) corresponding to 750 mOsmol·kg^{-1} (~25.5 PSU) and 820 mOsmol·kg^{-1} (~27.9 PSU) for *N. meinerti* and *T. urvillei*, respectively. Since results showed that iSW had no significant impact on bioenergetics, all further work was conducted under SW and dSW. Batch 3 was used to calculate osmoregulatory capacity and conduct gill morphometry and batch 4 was used to calculate Na^+/K^+-ATPase (NKA) labelling and activity. For the latter, anterior (with a mainly respiratory function) and posterior gills (with an important osmoregulatory role) were sampled separately. *Neosarmatium meinerti* possesses 8 gills per branchial cavity (Fig. 2D) while *T. urvillei* has 6 (Fig. 2E). The last three pairs in each species were considered as osmoregulatory gills, since this is classically reported in many decapod crabs.

Salinity for each experimental condition was controlled daily and adjusted if necessary during the different exposure times. For most crab species, full salinity acclimation occurs between 1-2 weeks of exposure (Lovett et al. 2006a). However, to remain within ecologically-relevant experimental times and to keep crabs in their intermolt stage (thus avoiding a change in their physiological responses), exposure was limited to 4 days, which is sufficient to achieve haemolymph osmoregulation (Siebers et al. 1972; Lovett et al. 2006b; Rivera-Ingraham et al. 2016a). For the morphological and NKA analyses, 7 days of exposure were allowed to ensure that morphological changes were efficiently induced (Tsai and Lin 2007). Additionally, to avoid the impact of feeding on the physiological parameters considered (Herreid 1980), crabs were fasted for a maximum period of 10 days during the experiments. Depending on the subsequent analyses and when possible, living animals were returned to their environment.

Osmolality curves

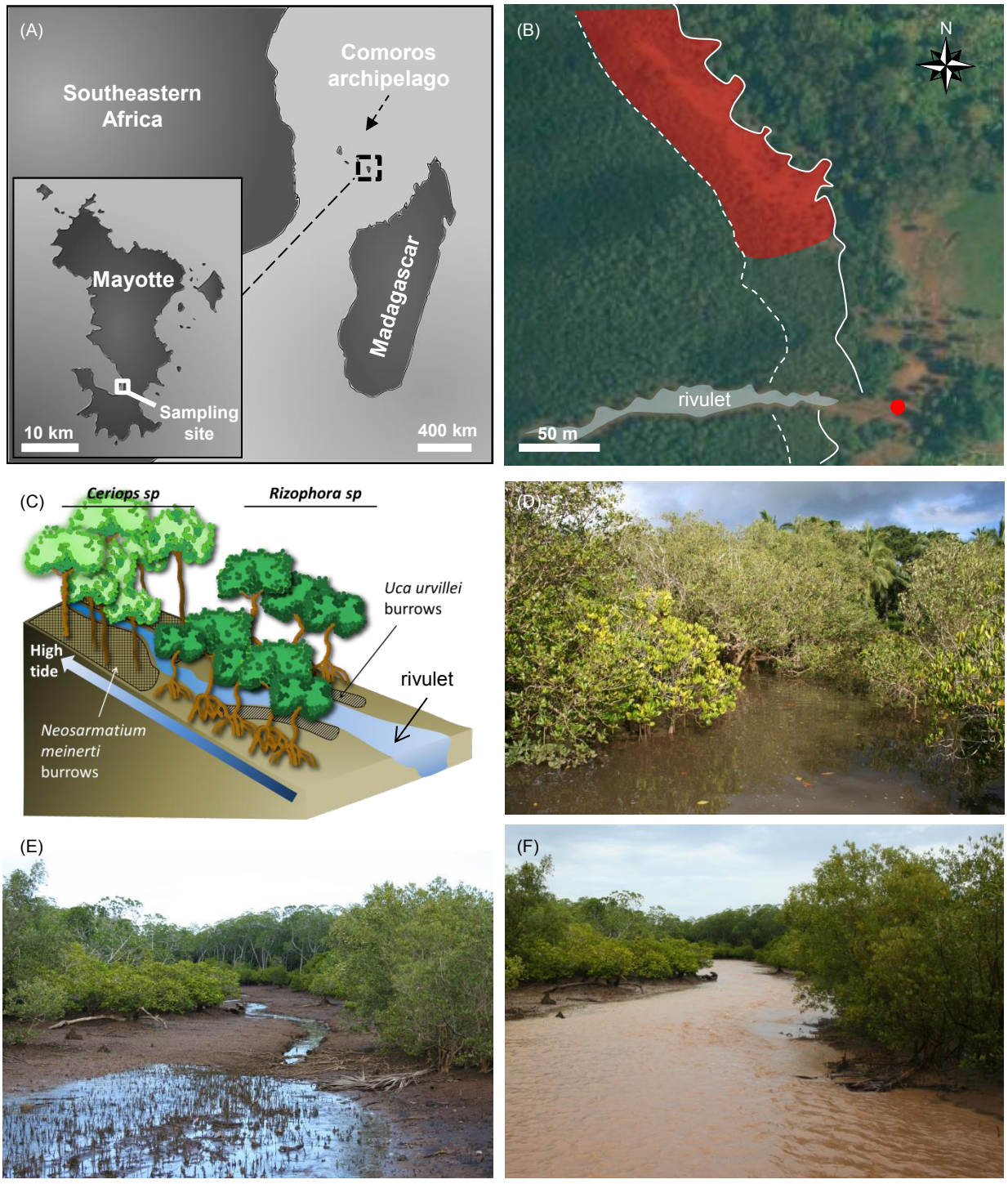

Fig. 1. Characteristics of the study site. (A) Location of the Comoros archipelago and Malamani mangrove (Mayotte Island). (B) Detailed view of the Malamani mangrove and *Neosarmatium meinerti* and *Tubuca urvillei* collection zones. Continuous line indicates the upper limit of the *Ceriops tagal* belt while the discontinuous line represents the approximate limit between the *C. tagal* and *Rhizophora mucronata* belts. The red dot corresponds to the position where pictures E and F were taken. Areas marked in red and blue represent the collection sites for *N. meinerti* and *T. urvillei*, respectively. (C) Schematic representation of the mangrove structure, representing the habitats of the two crab species. (D) General view of the mangrove (area marked in red in subpanel B) at high tide. E-F: General view of the rivulet at low tide during a dry day (E) and after rainfall (F).

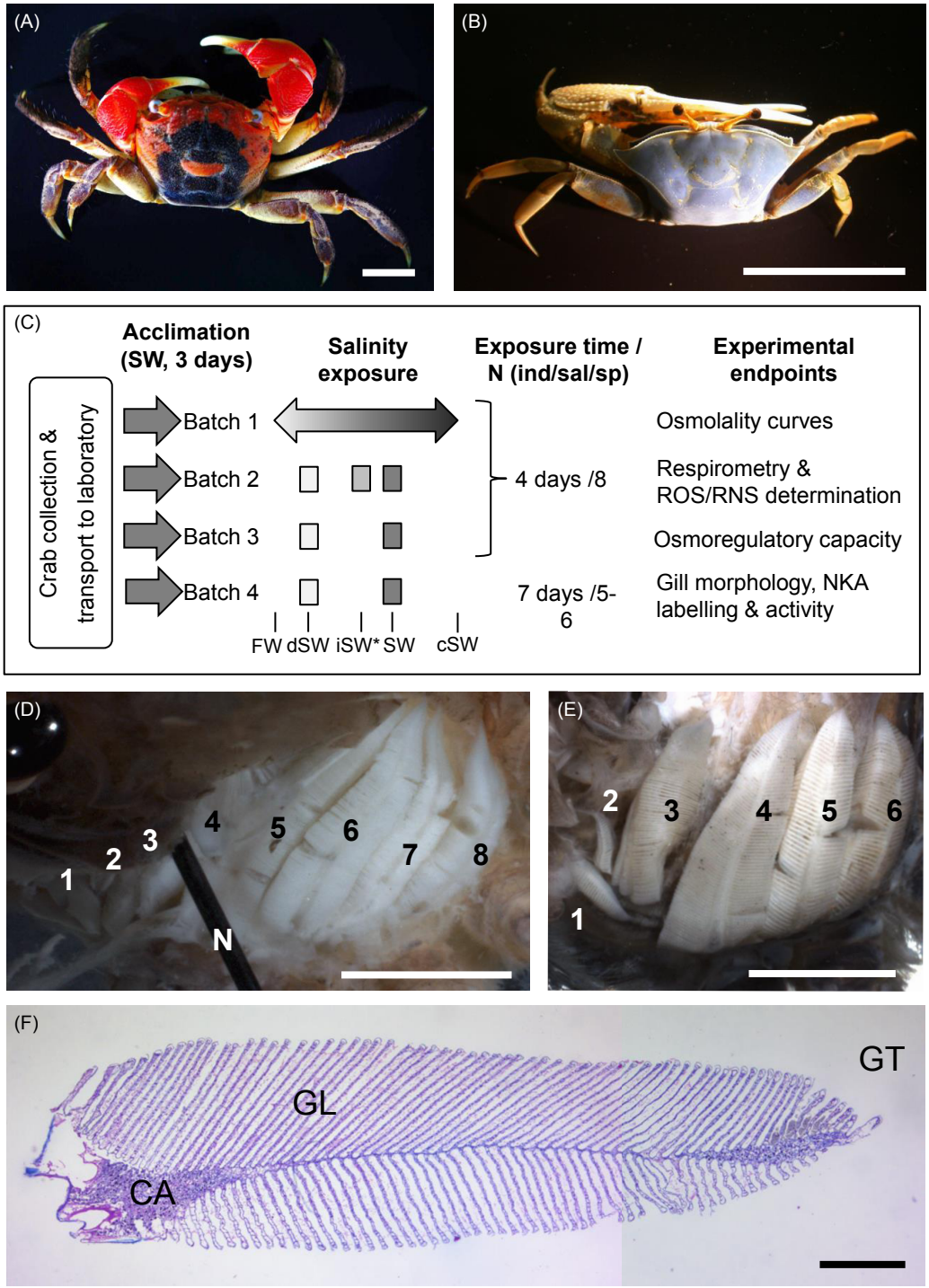

Fig. 2. Dorsal view of *Neosarmatium meinerti* (A) and *Tubuca urvillei* (B). (C) Schematic representation of the experimental setup. For those analyses considering gill tissues (morphology, NKA activity and labelling), anterior and posterior gills were considered separately. The macroscopic view of *N. meinerti* and *T. urvillei* gill cavities are shown in D and E, respectively. The branchiostegite covering the branchial cavity has been removed. Gills are numbered from the anterior side of the crabs. Both species present a similar gill organization but different total gill number: whilst there are 2 podobranchs and 4 arthrobranchs for *T. urvillei*, *N. meinerti* possess 3 podobranchs and 4 arthrobranchs. (F) Histological section of a phyllobranchiate gill (*T. urvillei*) with flat leaf-like lamellae connected to a central axis distributing and collecting the circulating hemolymph. Anterior and posterior gills of both species present a similar branchial organisation. CA: central axis; cSW: concentrated seawater; dSW: diluted seawater; FW: freshwater; GL: gill lamellae; GT: gill tip; iSW: isosmotic seawater; N: dissecting needle; SW: seawater. Scale bars: A = 3 cm, B = 2 cm; C = 2 cm; D = 7 mm; E = 500 µm. *values adjusted to the isosmotic point of each of the two species according to the results provided in figure 3A.

To determine osmolality curves for *N. meinerti* and *T. urvillei*, crabs were immersed at different water salinities with 8 crabs per salinity, ranging from FW (salinity ~0.2 PSU; 6 mOsmol·kg^{-1}) to concentrated SW (salinity ~50 PSU; 1500 mOsmol·kg^{-1}). Crabs were maintained in these conditions for 4 days, a typical acclimation time used for other decapods. A 0.1-0.5 ml hemolymph aliquot was then rapidly collected from crabs kept on ice using a 0.5 ml syringe without added anticoagulant, inserting the needle between the cephalothorax and a pereiopod. Hemolymph osmolality was measured in duplicate using a vapor pressure depression osmometer (Vapro 5600, Wescor, USA) (sample volumes: 10 μl).

Whole-animal respirometry

These experiments were conducted in 750 ml and 150 ml air-tight transparent chambers for *N. meinerti* and *T. urvillei*, respectively, equipped with magnetic stirrers to ensure correct O_2 mixing in the water column and equipped with an oxygen optode (OXSP5, sensor code SD7-545-214) (Pyro-Science GmbH, Aachen, Germany) on its inner wall. Optodes were calibrated to 100% (using air-saturated water) and 0% air saturation. The latter was achieved using a freshly made 80 mM Na_2SO_3 solution. A structure of 1 mm^2 mesh was installed in the chamber to avoid the disturbance of crabs by the magnetic stirrer (Rivera-Ingraham et al. 2016a). Each crab was placed in a chamber containing filtered (0.2 μm Whatman) medium at the salinity value at which the animal was acclimated. To avoid handling stress, crabs were left for 15 min with an aeration system to maintain fully oxygenated water. Each chamber was then filled to its maximum capacity to avoid the formation of air bubbles and was sealed to ensure air tightness. Oxygen concentration within each chamber was measured using a four-channel fiber-optic oxygen meter (FireSting, Pyro-Science GmbH) and was recorded through the Pyro Oxygen Logger software. All measurements started in fully oxygenated water (> 98 %) and oxygen consumption rates were recorded as a function of declining pO_2 over time. Four parallel measurements were carried out simultaneously (including a blank) and data were recorded at 5 s intervals. Animals in each of the chambers were allowed to breathe for a minimum of 15 min at a constant and controlled temperature of 25°C. After each experiment, crabs were blotted dry and fresh body weight (FBW) was recorded to the nearest 0.01 g. Rates of oxygen consumption (MO_2) were calculated through linear regression and expressed as μmol O_2·min^{-1}·g FBW^{-1}.

ROS/RNS formation

For each experimental condition (SW, iSW and dSW), 8 crabs were individually weighed (to the nearest 0.01 g) before hemolymph sampling. Hemolymph samples were collected for each individual, as previously described. The presence of ROS/RNS in the hemolymph of mangrove and fiddler crabs was performed using the spectrophotometric procedure described by Rivera-Ingraham et al. (2016a). In triplicate, 2 μl of hemolymph sample was mixed with 100 μl of a saline solution. To avoid osmotic-induced ROS/RNS production by hemocytes, the osmolality of the solution was adjusted to the osmolality of the hemolymph. According to the osmolality curves, these values were: 649, 690 and 765 mOsmol·kg^{-1} for *N. meinerti*, and 765, 780 and 874 mOsmol·kg^{-1} for *T. urvillei* for the SW, iSW and dSW treatments, respectively. Reactions were initiated with 50 μl of Dichloro-dihydro-fluorescein diacetate solution (DCFH-DA, 30 μM) (Molecular Probes, C-13293). The fluorescence signal was read at wavelengths of 590 nm (excitation) and 510 nm (emission) using a Tecan infinit F200 PRO (Tecan Group Ltd., Austria).

Measurements were made every minute over a 20 min period in a flat-bottom black microplate. The speed of DCFH-DA degradation in DCF is proportional to the content in ROS/RNS. As DCF fluorescence may vary according to the salinity of the medium (Rivera-Ingraham et al. 2016a), a standardization curve of H_2O_2 was prepared and measured at each of the 6 environmental salinities (3 per species) to correct any such differences, as described by Rivera-Ingraham (2016a). Although it has been widely demonstrated that DCF is not H_2O_2-specific for various reasons (Grisham 2012; Kalyanaraman et al. 2012), the curve was constructed using this compound as it is the most stable, and thus the most common, ROS present within cells.

Osmoregulatory capacities

The osmoregulatory capacity (OC) of *N. meinerti* and *T. urvillei* was assessed by recording hemolymph osmolality after 0, 1, 2 and 4 days of exposure to either dSW or SW. Each crab was sampled once. The OC was calculated as the absolute value of the difference between the

osmotic pressure of the hemolymph and that of the external medium.

Branchial histology, immunolabelling of the Na^+/K^+-ATPase (NKA) and morphometric analyses

A total of 5 crabs per species were exposed to SW and dSW for a period of 7 days to ensure that all anatomical changes occurred fully. After this period, animals were anesthetized on ice and sacrificed. Anterior and posterior gills were sampled separately. For *N. meinerti*, anterior gill pair 1 and posterior gill pair 8 were sampled. For *T. urvillei*, anterior gill pair 3 and posterior gill pair 6 were used. Dissected gills were fixed in Bouin's fixative solution for 2 days and subsequently rinsed in 70% ethanol. Samples were then dehydrated in a series of graded alcohols (90, 95 and 100%) and embedded in paraffin. Sections (4 μm) were cut on a Leitz Wetzlar microtome, collected on glass slides and stained using the classical Masson's Trichrome Staining Protocol (Martoja and Martoja-Pierson 1967) or directly used for immunolabelling.

For NKA immunolabelling, sections were dewaxed; hydrated through a graded series of ethanol (90, 95, 100%); rinsed with a solution of 10 mM Phosphate-buffered saline (PBS), 150 mM NaCl and 0.01% Tween 20 (pH 7.3), for 10 min; then treated with 50 mmol NH4Cl in PBS (pH 7.3) for 5 min to mask free aldehyde groups in the fixative. Finally, sections were incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatine in PBS. Primary labelling was performed for 2 h at room temperature in a wet chamber, with the primary antibody rabbit anti-NKA H300 (Santa Cruz Biotechnology) diluted in PBS at 10 $\mu g \cdot ml^{-1}$ covering the sections. Control sections were incubated in BS without the primary antibodies. After 3 extensive 10 min washes in BS to remove unbound antibodies, the sections were incubated for 1 h with a secondary antibody: donkey-anti rabbit (Alexa Fluor® 546), 10 $\mu g \cdot ml^{-1}$. Following extensive washing in BS (six times for 5 min), sections were mounted in 80% glycerine, 20% PBS and 2% N-propyl-gallate to delay photobleaching (ImmunoHistoMount, Aqueous-based Media, Santa Cruz Bio-technology, USA).

Stained and labelled sections were examined with a Leitz Diaplan microscope equipped with a special filter for fluorescence and associated with a Leica DC 300 F digital camera and its software FW 4000 I (Leica Microsystems, Rueil-Malmaison, France).

Histological sections of the gill lamellae were photographed at the same magnification (×40). The thickness of the gill epithelium was measured on both sides of 5 lamellae per gill (only areas sectioned through cell nucleus were considered). Morphometric measures were obtained using the freeware Image J (http://rsbweb.nih.gov/ij/).

Branchial NKA activity

To identify quantitative differences in NKA activity between the species, an additional 6 crabs per species were exposed to the same conditions described in section 2.7. The last three pairs of posterior gills (6, 7 and 8 for *N. meinerti* and 4, 5 and 6 for *T. urvillei*) were dissected and homogenized in pairs in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.4) using a manual potter. The specific, Na^+- and K^+-dependent, ouabain-sensitive ATPase activity was measured in these homogenates using 1.4 mM of ouabain as described in detail by Metz et al. (2003). Aliquots (5 μl in triplicate) of homogenate (protein content of 1 $mg \cdot ml^{-1}$) were incubated in assay media for 20 min at 25°C. The specific activity was calculated by subtracting the K^+-independent, ouabain-insensitive ATPase activity from total ATPase activity. ATP hydrolysis was assessed by the amount of inorganic phosphate formed per minute per mg of protein. Sample protein content was estimated using the protocol originally described by Bradford (1976) using a commercially prepared reagent (Sigma-Aldrich, Saint Louis, USA), and bovine serum albumin as standard.

Statistics

Statistical analyses were conducted using R software (R Core Team 2016) and SPSS 15.0 (SPSS Inc., USA). The R statistic package 'cobs' was used to adjust the osmolality curves. This package is based on constrained b-spline approximations (Ng and Maechler 2007). T-tests and one- or two-way ANOVAs were carried out on data after control of parametric test assumptions (normality and homoscedasticity). When a significant difference was detected, differences among groups were identified using Tukey's Honest Significant Difference (HSD) test for two-way ANOVA and Student-Newman-Keuls (SNK) test for one-way ANOVA. Data are presented as means with standard error of the mean (± SE) and the level of statistical significance was $p < 0.05$.

RESULTS

For all analyses, no significant differences were found between treatments in terms of animal weight. In the case of *N. meinerti*, there was no influence of sex on any of the parameters analyzed and thus only pooled results are shown. The salinity range measured during crab sampling in the collection zones of *N. meinerti* and *T. urvillei* (Fig. 1B, C) was 911-1352 mOsm·kg^{-1} (31-46 PSU) and 273-521 mOsm·kg^{-1} (10-18 PSU), respectively (Fig. 3A).

Hemolymph osmolality curves, salinity tolerance and osmoregulatory capacity (OC)

Both *N. meinerti* and *T. urvillei* are strong osmoregulators across the tested salinities (Fig. 3A). Crabs hyper-regulate at lower salinities (hemolymph osmolality is actively maintained

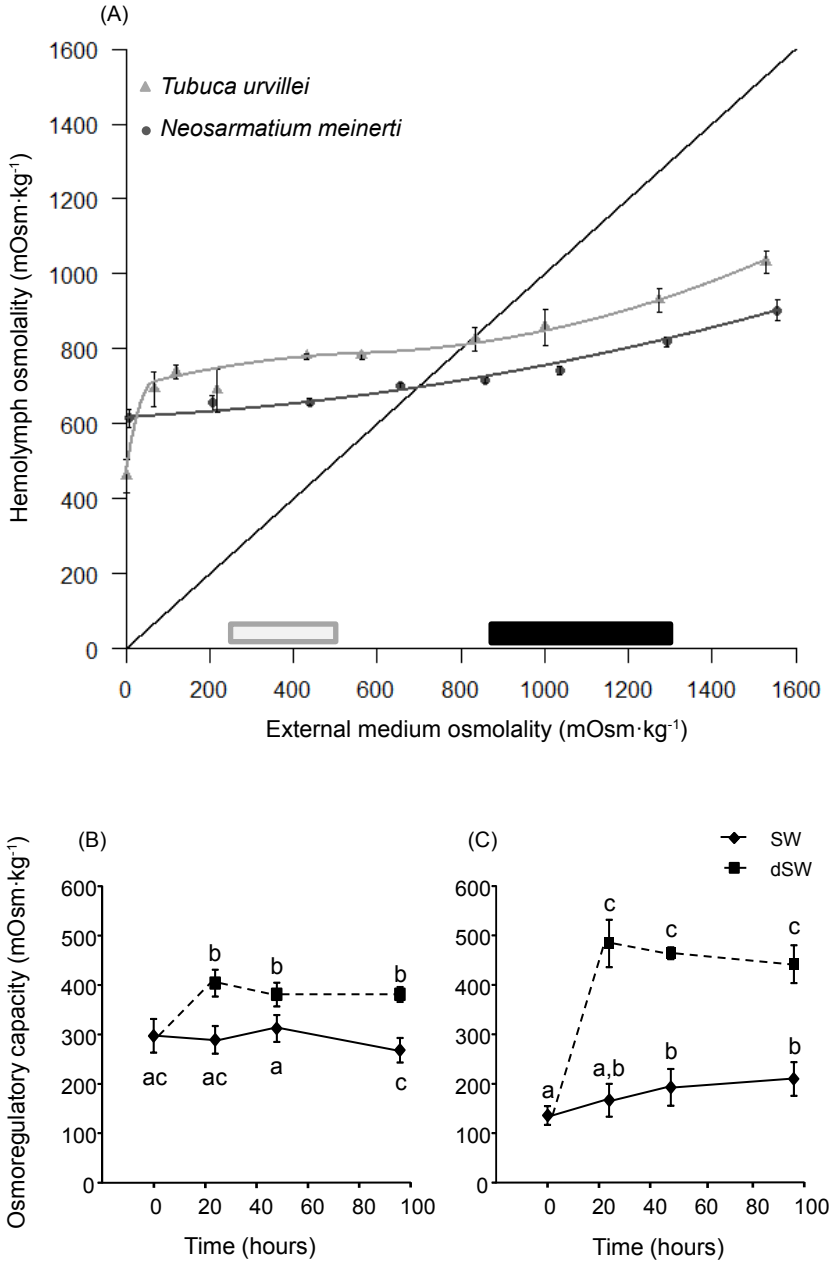

Fig. 3. Hemolymph osmolality (in mOsm·kg^{-1}) at different salinities (A) and osmoregulatory capacity after salinity challenge (B, C) shown by *Neosarmatium meinerti* (B) and *Tubuca urvillei* (C). Grey bar: salinity range of water collected at low tide along the rivulet banks. Black bar: salinity range of water collected at low tide in burrows of *N. meinerti*.

at levels above that of dSW) but show a hypo-osmoregulatory behaviour at higher salinity (hemolymph osmolality maintained below that of SW). They can maintain a gradient greater than 600 mOsm·kg^{-1} when exposed to salinities close to FW (5-10 mOsm·kg^{-1}) or concentrated SW (1500 mOsm·kg^{-1}). The only deaths recorded at the lowest and highest salinities were in *T. urvillei*. Mortality rates were up to 37 % at 5 mOsm·kg^{-1} and 12% at 1500 mOsm·kg^{-1}.

For crabs kept in SW or directly transferred to dSW, OC data after 4 days post transfer confirms these crabs are strong osmoregulators and can maintain a stable osmoregulation within hours after transfer (Fig. 3A, 3B). *Neosarmatium meinerti* can maintain OC values of ~300 mOsm·kg^{-1} in SW (30 PSU) and ~400 mOsm·kg^{-1} in dSW (10 PSU) over time, and these values were significantly different ($F = 34.54$, $p < 0.001$) (Fig. 3B). The same is true for *T. urvillei* ($F = 174.28$, $p < 0.001$), which also presents remarkable regulatory capacity with OC values of ~200 mOsm·kg^{-1} in SW (30 PSU) and ~500 mOsm·kg^{-1} in dSW (10 PSU) (Fig. 3C).

Rates of oxygen consumption and ROS/RNS production

Oxygen consumption rate across salinities did not vary for *N. meinerti* ($F = 0.52$, $p \approx 0.59$, Fig. 4A) or for *T. urvillei* ($F = 0.05$, $p \approx 0.95$, Fig. 4B). ROS/RNS production in the hemolymph (Fig. 4C and D) did not show any significant difference for either species ($F = 0.48$, $p \approx 0.62$ and $F = 1.01$, $p \approx 0.38$ for *N. meinerti* and *T. urvillei*, respectively).

Branchial histology

The histological analysis revealed relevant differences among anterior and posterior gills in both species. No discernible differences

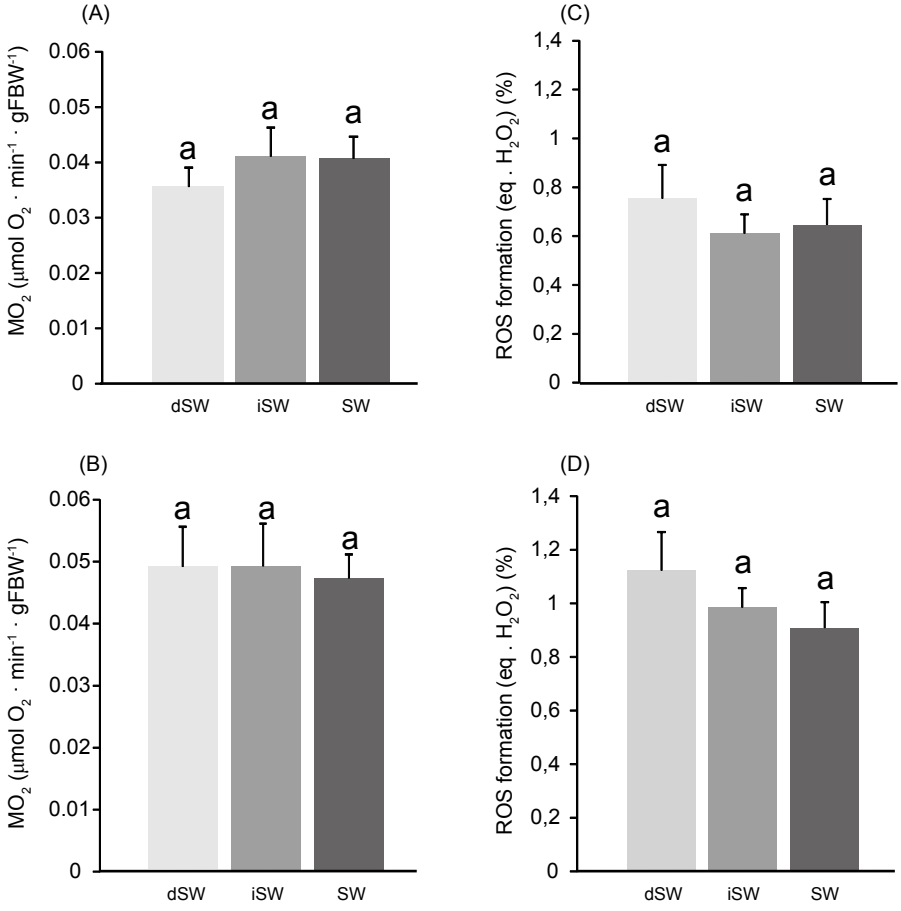

Fig. 4. Whole animal rates of oxygen consumption (A, B) and ROS/RNS production in hemolymph (C, D) from *Neosarmatium meinerti* (A, C) and *Tubuca urvillei* (B, D). Different letters represent significantly different groups according to a one-way ANOVA test followed by a SNK multiple comparison test ($p < 0.05$). iSW: isosmotic SW (750 mOsm·kg^{-1} for *N. meinerti* and 820 mOsm·kg^{-1} for *T. urvillei*). FWB: fresh body weight.

in morphology were found between animals acclimated to SW and those exposed to dSW. Thus, only results corresponding to the latter are shown. In both cases, anterior gills showed the typical characteristics of respiratory organs: gill lamellae consist of a single layer of very thin epithelial cells while the hemolymphatic space is divided into upper and lower sections by a septum (Fig. 5A, B). Posterior gills do not have this septum and present a much thicker epithelial layer. Measurements of epithelial thickness on histological sections show large differences between anterior and posterior gills (Fig. 5E, F), and it is especially striking that values are over 2-fold higher for the small *T. urvillei.*

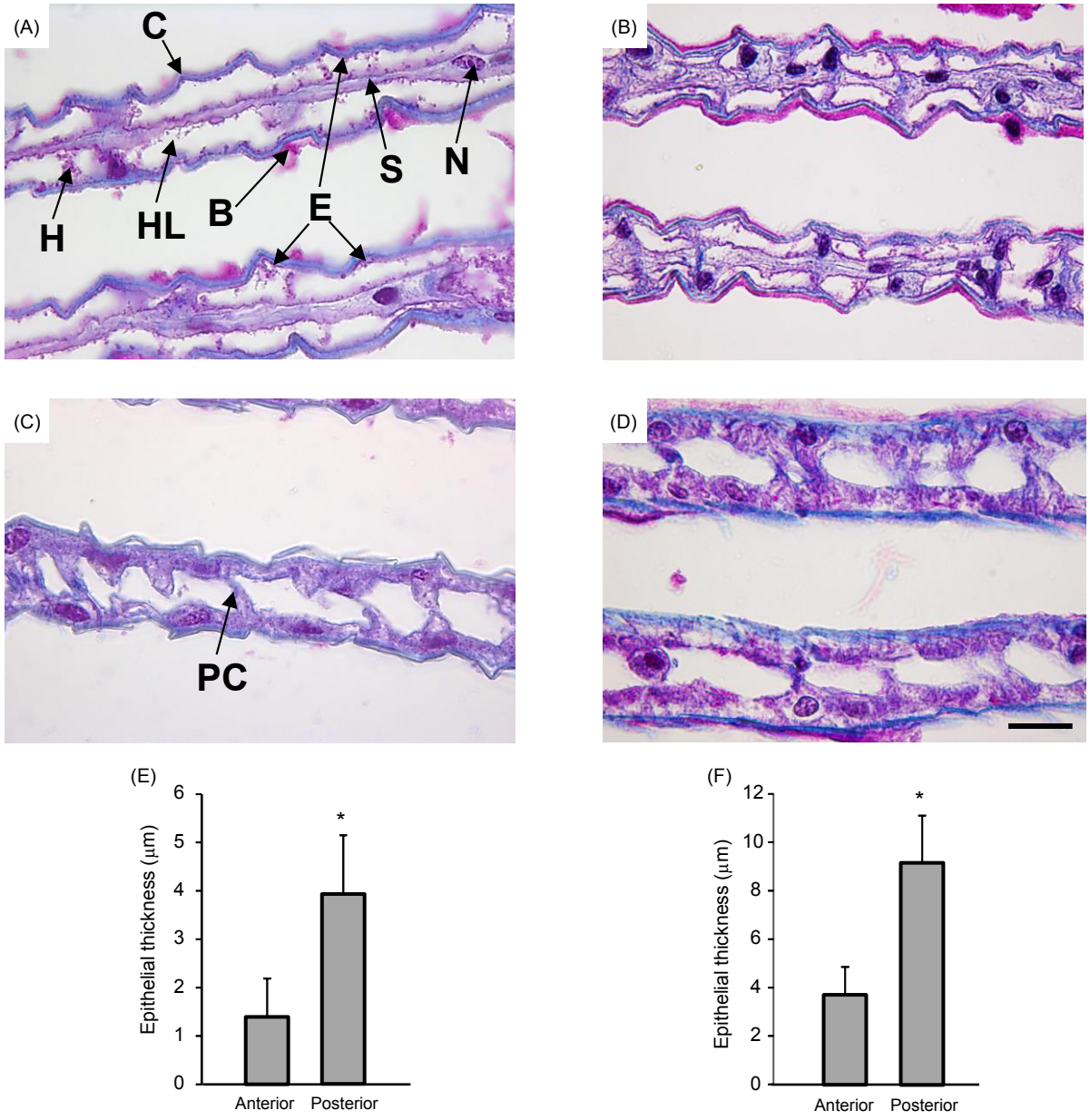

Fig. 5. Histological sections (A to D) and epithelial thickness of gill lamellae (E, F) of *Neosarmatium meinerti* (A, C, E) and *Tubuca urvillei* (B, D, F) after dSW acclimation. Subpanels A and B correspond to anterior (mainly respiratory) gills (here pairs 1 and 3 for *N. meinerti* and *T. urvillei*, respectively). Subpanels C and D correspond to posterior (osmoregulatory) gills (here, pairs 8 and 6 for *N. meinerti* and *T. urvillei*, respectively). B: bacterial layer; C: cuticle; E: epithelium; H: hemocyte; HL: hemolymphatic lacuna; N: nucleus; S: septum. Scale bar = 20 μm. * $p < 0.001$ (*t*-test).

Branchial Na^+/K^+-ATPase labelling and activity

NKA immunolocalization conducted on histological sections of both species (Fig. 6) indicates that the labelling occurs along the basal side of the epithelial cells and appears reduced in the anterior gills (Fig. 6A, B), but immunostaining is thicker in the posterior gills (Fig. 6C, D) in both species.

For *N. meinerti*, the two-way ANOVA

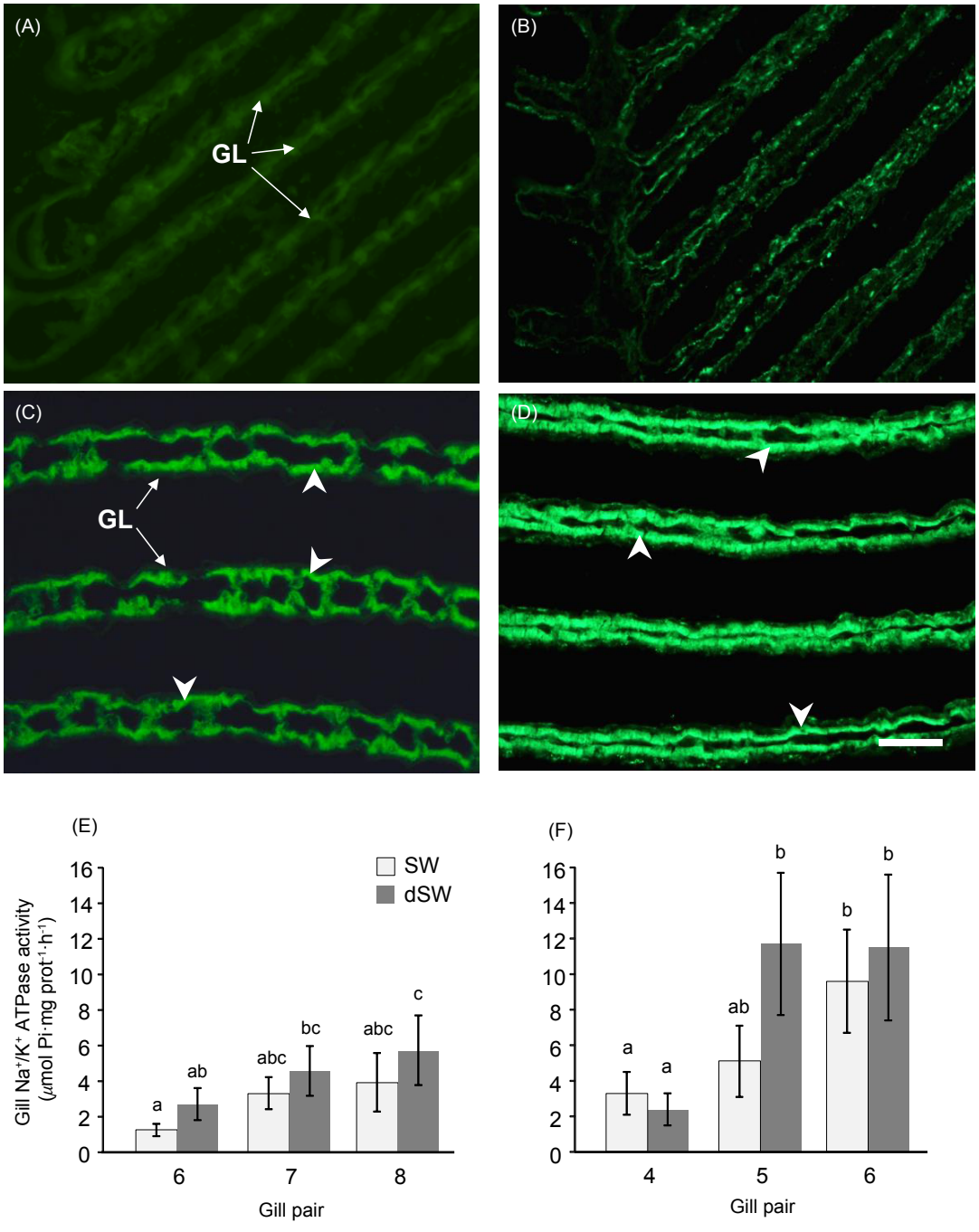

Fig. 6. NKA immunolabelling and activity for *Neosarmatium meinerti* (A, C, E) and *Tubuca urvillei* (B, D, F). NKA immunolabelling is shown for anterior (A, C) and posterior (B, D) gills (here, gill 8 for *N. meinerti* and gill 6 for *T. urvillei*). NKA activity (E, F) was measured from the last three gill pairs of crabs exposed to SW or dSW. Different letters show statistically significant differences based on a two-way ANOVA followed by Tukey's HSD test ($p < 0.05$). GL: gill lamellae; arrow heads indicate NKA labelling. This labelling occurs along the basal side of the epithelial cells and appears reduced in the anterior gills but thicker in the posterior gills. Scale bar = 40 μm.

revealed that NKA activity is significantly different between the three posterior gills ($F = 11.154$; $p < 0.001$), with a salinity effect ($F = 5.761$; $p < 0.05$). However, post-hoc analysis did not reveal any difference between SW and dSW crabs among gill pairs (Fig. 6E).

For *T. urvillei*, no difference was observed in NKA activity recorded in the last three posterior gills between SW- and dSW-exposed crabs (two-way ANOVA, $F = 3.316$; $p = 0.08$) but there was a significant difference according to the position of the gills ($F = 21.149$; $p < 0.001$). NKA gill activity increased by up to 6 times between gill pairs 4 and 6 (Fig. 6F).

DISCUSSION

In a mangrove ecosystem, the key factors driving the spatial and temporal abundance of amphibious sesarmid and fiddler crabs are still not fully understood (Koch et al. 2005; Bezerra et al. 2006; Salgado-Kent and McGuinness 2010; Nobbs and Blamires 2015). In the mangrove site of Mayotte, Comoros archipelago, the fiddler crab *Tubuca urvillei* and the red mangrove crab *N. meinerti* are both strong osmoregulators, but they live in different areas of the upper mangrove and thus face different salinity ranges. *N. meinerti* is periodically subjected to extreme salinity fluctuations and regularly faces high salinity water in its burrows, most probably due to SW evaporation at low tide and infrequent seawater renewal at high tide, depending on the tidal coefficients (this study; Gillikin et al. 2004). Fiddler crabs; however, live preferentially close to flowing brackish water, *i.e.* close to rivulets draining freshwater from the mainland (this study; Nobbs and Blamires 2015) and, unlike *N. meinerti*, use a burrow plugging behavior (Fusi et al. 2015), thereby minimizing their exposure to wide salinity variations.

Existing literature suggests that mangrove crab osmoregulatory ability is not strongly linked with observed zonation patterns, species abundance and distribution (Frusher et al. 1994; Gillikin et al. 2004). Our results support this hypothesis. From a purely energetic perspective, this is shown by the rates of oxygen consumption recorded for the two species. Both species maintain relatively stable oxygen consumption rates across different salinities, suggesting that shifts in environmental salinity do not impose a significant change in energy demand or entail a redox imbalance. However, the different subtle physiological characteristics recorded in this study widen our knowledge of the ecological *in-situ* salinities observed for fiddler and red mangrove crabs. *Neosarmatium meinerti* has a strong hypo-osmoregulatory capacity (hypo-OC) in SW and hyper-OC in dSW, indicating that this species has a strong OC under these conditions (Lignot et al. 2000; Henry et al. 2012). *Tubuca urvillei* shows a high hyper-OC at the lower salinity levels it encounters in its habitat, and a comparatively low hypo-OC in SW. This OC shift at low salinities further supports the conclusion that high salinity conditions are a larger physiological challenge for *T. urvillei* than *N. meinerti.* This is consistent with NKA gill activity recorded in the posterior osmoregulatory gills of crabs maintained in SW and dSW. NKA activity is up to 6 and 12 μmol Pi·mg $prot^{-1}·h^{-1}$ for *N. meinerti* and *T. urvillei*, respectively. Also, the osmoregulatory epithelium of the posterior gill lamellae is up to 4 μm thick for *N. meinerti* and 9 μm for *T. urvillei*. Similar osmoregulatory gill epithelium thicknesses were also observed in hololimnetic crabs such as *Dilocarcinus pagei* (Furriel et al. 2010) and *Potamon niloticus* (Maina 1990). In other true freshwater species such as crayfish (*i.e. Astacus pallipes*, *Procambarus clarkii* and *Orconectes virilis*), the water-blood barrier thickness can also measure up to 8 μm (Fisher 1972; Burggren et al. 1974; McMahon et al. 1974), even attaining 18-20 μm in the Trichodactylid crab *Dilocarcinus pagei* (Onken and McNamara 2002). A similar thickness to that obtained for *T. urvillei* was measured in *Leptuca uruguayensis* (Luquet et al. 1995). Terrestrial Gecarcinidae, Grapsidae, Ocypodoidea and Sundathelphusidae crustaceans (*i.e. Geograpsus grayi*, *G. crinipes*, *Cardisoma hirtipes*, *Gecarcoidea natalis*, *Mictyris longicarpus* and *Holthuisana transversa*) also have thick osmoregulatory epithelia of up to 10 μm in their posterior gills (Taylor and Greenaway 1979; Farrelly and Greenaway 1992). Consequently, the blood/gas diffusion distance limits the efficiency and absolute contribution of the gills to gas exchange. The presence of well-developed epithelia in posterior gill lamellae in fiddler and red mangrove crabs is therefore typical of FW, amphibious and land crabs. This represents one of the major morphological evolutions towards terrestrialization.

Finally, osmoregulatory posterior gills of fiddler and red mangrove crabs do not suffer any major morphological changes due to dSW transfer. This is not the case for most intertidal

euryhaline crustaceans, for which the need for increased ion transport during low-salinity transfer drives epithelial hypertrophy in the osmoregulatory gill lamellae through various and well-studied mechanisms (Neufeld et al. 1980; Henry and Watts 2001; Roy et al. 2007; Torres et al. 2007; Lucu et al. 2008). All these changes are linked to an increase in NKA-specific activity along with increased expression of other ion transporters and supporting enzymes such as carbonic anhydrase, $Na^+/K^+/2Cl^-$ cotransporter, V-type H^+-ATPase and arginine kinase (Pequeux 1995; Lucu and Towle 2003). However, no salinity-induced changes in NKA activity occur for bimodal and terrestrial crabs (Tsai and Lin 2007), as observed for *N. meinerti* and *T. urvillei* in our study. A possible explanation is that the cellular machinery is already optimized for terrestrial life, with the involvement of posterior gills in acid-base balance, nitrogen excretion and aerial osmoregulation. This machinery could thus easily switch to aquatic osmoregulation with no functional and morphological remodeling of the gills. It could involve specific enzymes such as the apical-type V-H^+-ATPase which, along with NKA, is a key enzyme in the transition from a marine environment to land and is involved in acid-base balance and nitrogen excretion (Morris 2001; Weihrauch et al. 2001 2004). As already demonstrated by Tsai and Lin (2007), intertidal crabs with varying NKA activity show a cytoplasmic V-H^+-ATPase distribution due to salinity challenge, but freshwater and terrestrial crabs with stable NKA activity during salinity variations tend to have an apical V-H^+-ATPase. It would therefore be interesting to investigate V-H^+-ATPase expression and localization in *T. urvillei* and *N. meinerti* to further characterize their adaptation to freshwater and terrestrial habitats. It would also be relevant to analyze the morphology of extrabranchial organs such as the branchiostegite, which has an osmoregulatory function in FW decapod crayfish (Lignot et al. 2005). This organ is also used for aerial oxygen uptake, since it has a reduced epithelial diffusion distance as low as 2 μm (Halperin et al. 2000). Finally, to better comprehend adaptation to terrestrial environments, it would be interesting to consider the ability of fiddler and red mangrove crabs to control their urine composition. Terrestrial crabs normally have access to FW but not to SW, and must therefore recover salt from their urine. This mechanism down-regulates a normally active uptake system, making it more suitable for their ecology.

CONCLUSIONS

We applied integrative physiological tools to provide new insights into the factors affecting crab distribution and, in doing so, add to the field of mangrove ecophysiology. Although salinity alone does not explain the spatio-temporal distribution of mangrove crabs, this study highlights that salinity-induced physiological constraints partly shape the distribution of crabs across a range of habitats within their salinity range. This physiological approach is a potential tool for assessing the impacts of salinity variations induced by human activities on mangrove crab distribution. This is essential to understand mangrove crab distribution and to predict how their disappearance could affect the functionality of mangrove ecosystems (Nobbs and Blamires 2015). This is particularly relevant since, among other practices, the release of domestic wastewater (with salinities close to FW values) or effluents from shrimp farms (with salinities close to SW values) may become a common practice in many countries that use mangrove forests as a natural filtering system (Wong et al. 1997; Ouyang and Guo 2016 2018). Mangrove crabs are also key players in the bioremediation process through their bioturbation activities.

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